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Sent: Tuesday, January 08, 2002 5:57 PM
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L14 ANSWER 1 OF 10 MEDLINE
AN 2000065523 MEDLINE
DN 20065523 PubMed ID: 10597631
TI ***Protein*** ***disulfide*** ***isomerase*** : the multifunctional redox chaperone of the endoplasmic reticulum.
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SO SEMINARS IN CELL AND DEVELOPMENTAL BIOLOGY, (1999 Oct) 10 (5) 481-93.
Ref: 111
Journal code: C2B; 9607332. ISSN: 1084-9521.

seminars in **CELL & DEVELOPMENTAL BIOLOGY**, Vol 10, 1999: pp. 481–493
Article No. scdb.1999.0319, available online at http://www.idealibrary.com on IDEAL®

Protein disulfide isomerase: The multifunctional redox chaperone of the endoplasmic reticulum

*Robert Noiva**

Protein disulfide isomerase (PDI) is a protein-thiol oxidoreductase that catalyzes the oxidation, reduction and isomerization of protein disulfides. In the endoplasmic reticulum PDI catalyzes both the oxidation and isomerization of disulfides on nascent polypeptides. Under the reducing condition of the cytoplasm, endosomes and cell surface, PDI catalyzes the reduction of protein disulfides. At those locations, PDI has been demonstrated to participate in the regulation of receptor function, cell–cell interaction, gene expression, and actin filament polymerization. These activities of PDI will be discussed, as well as its activity as a chaperone and subunit of prolyl 4-hydroxylase and microsomal triglyceride transfer protein.

Key words: endoplasmic reticulum / prolyl 4-hydroxylase / protein disulfide isomerase / protein-thiol oxidoreductase / microsomal triglyceride transfer protein

Introduction

THE FOLDING AND ASSEMBLY of secretory and membrane proteins is a complicated process that is initiated in the lumen of the rough endoplasmic reticulum (ER). The environment of the ER lumen provides the pH and redox conditions required for folding, as well as access to chaperones and foldases. A particularly interesting ER foldase is the protein-thiol oxidoreductase, protein disulfide isomerase (PDI; EC 5.3.4.1). PDI was first characterized by the pioneer of protein folding, Christian Anfinsen, based upon its ability to catalyze the refolding of ribonuclease A, an enzyme with 4 disulfide bridges.[1] The protein-thiol oxidoreductase activity of PDI appears to differ based on the redox environment and cellular requirements. PDI catalyzes the oxidation of disulfides and isomerizes incorrect (or correct) disulfides on nascent polypeptides undergoing folding in the oxidizing environment of the ER. Additionally, PDI is present in other cellular locations in many cell types. PDI reduces disulfide bridges of proteins in the cytosol, endosomes, and at the plasma membrane as a consequence of the more reducing environments of those locales. Although PDI was initially characterized as an oxidoreductase, we now know it is a multifunctional participant in the folding, assembly, and post-translational modification of many proteins. PDI is a subunit of two well characterized ER enzymes, prolyl 4-hydroxylase (P4H; EC 1.14.11.2) and microsomal triglyceride transfer protein (MTP). Although the role of PDI in those other enzyme complexes is not well understood, PDI is known to play an essential role in their function. Like other chaperones and foldases, PDI expression appears to be related to events, such as heat shock, which increase the need for chaperones in the cell.

The purpose of this review is to provide basic background to the reader in the structure–function relationships of the PDI enzyme and to newer areas of active investigation into PDI function. Numerous reports have identified additional roles of PDI in cellular function outside of the folding, assembly, and post-translational modification of polypeptides in the ER. PDI localized to plasma membranes of many different cell types has been clearly demonstrated to have important physiological function. It is important to critically discuss these additional PDI functions

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; MTP, microsomal triglyceride transfer protein; P4H, prolyl 4-hydroxylase; PDI, protein disulfide isomerase; PMN, polymorphonuclear neutrophils; RNase A, ribonuclease A

1084-9521/99/050481+13 $30.00/0

and to fit the multiple roles of this interesting enzyme into a more global scheme. In this review, current ideas about the function, subcellular localization, and regulation of expression of PDI will be discussed.

PDI structure

PDI belongs to the superfamily of protein-thiol oxidoreductase enzymes with sequence and structural similarity to thioredoxin. The primary sequence of PDI as deduced from the PDI cDNA first indicated its inclusion in the superfamily of oxidoreductases because of its extensive sequence similarity to thioredoxin in the region of the two protein-thiol oxidoreductase active sites.[2] Analysis of the primary structure, intron/exon boundaries, limited proteolysis, NMR, and computer modeling of the PDI enzyme has led to a five domain model for the enzyme (Figure 1).[3] PDI is composed of four domains with thioredoxin folds (a–b–b′–a′), followed by a c region that probably does not have secondary or tertiary structure. There is extensive internal sequence similarity between the a and a′ domains as well as between the b and b′ domains. The two protein-thiol oxidoreductase active site sequences of PDI (-Cys-Gly-His-Cys-) are located in the a and a′ domains.

Although high resolution X-ray crystal structure of PDI has not been determined, the recent structural determination of a disulfide isomerase from the archaeon *Pyrocccus furiosus* may be useful in the prediction of structure–function relationships of the a domain of PDI. This archaeon PDI contains two domains, each with extensive sequence similarity to the a and a′ domains of mammalian PDI, and like mammalian PDI, is active as a homodimer.[4] The crystal structure of archaeon PDI and NMR studies of the a domain of PDI[5] indicate it has a tertiary structure similar to *E. coli* thioredoxin and dsbA. The a domain of PDI has a thioredoxin fold with two grooves that are probable sites of enzyme–substrate interaction. Recent NMR studies of the b domain of PDI indicate that like the a and a′ domains, the b domain has a thioredoxin fold[6] despite the absence of any Cys-Xaa-Xaa-Cys- redox active sites or sequence similarity to thioredoxin. Although high resolution structural analyses of the a′ and b′ domains have not been performed, their high degree of sequence similarity to the a and b domains suggest they are likely to have similar structures. Therefore, the proposed structure of PDI is a total of four domains with thioredoxin-like folds, two with redox active sites, and two with no redox activity.

The c domain has been defined as the region from amino acid residue 465 to the C-terminus of the PDI polypeptide. This region of PDI may not be an actual domain as there is no evidence of defined secondary or tertiary structure. However, the c 'domain' is of considerable interest because of its high content of acidic residues and the presence of the C-terminal -KDEL ER retention signal. The C-terminal -KDEL motif of the PDI polypeptide is involved in the ER localization of the enzyme. The acidic residues of the c domain participate in the extensive calcium binding activity of PDI (discussed below).

Recent reports of the determination of the structure of MTP by X-ray diffraction may also contribute to our understanding of the structure of PDI.[7] PDI is the smaller subunit of the MTP $\alpha\beta$ heterodimeric complex. The MTP structure, as determined using X-ray diffraction to a resolution of 3 Å, predicts PDI to have four distinct domains surrounding a clamshell-like large subunit. In the MTP structure heterodimer, the four regions of PDI (a–b–b′–a′) are clearly identifiable as distinct domains, but do not appear to interact with one another. However, since PDI is active as a homodimer, domain interactions

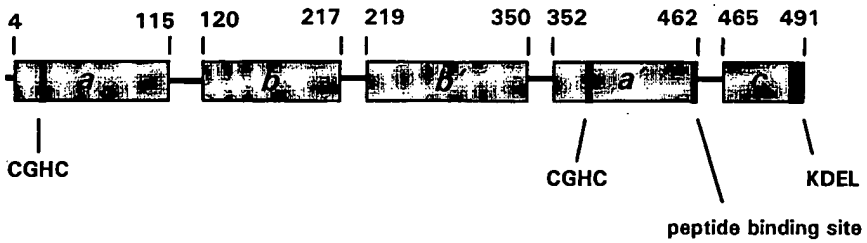

Figure 1. The structure of mammalian PDI. Domain boundaries as proposed by Darby *et al*[27] are noted. The location of the proposed peptide binding site and the C-terminal -KDEL ER retention signal are also depicted.

(a–a, b–b, or a–b) between the two polypeptides may occur.

PDI oxidoreductase activity

PDI belongs to a subset of the protein thiol-disulfide oxidoreductases with sequence similarity to thioredoxin that catalyze the oxidation or reduction of disulfide bridges. This family of thioredoxin-like enzymes includes prokaryotic enzymes, such as DsbA, eukaryotic enzymes, such as Erp72, CABP1, and PDI, as well as the ubiquitous enzymes thioredoxin and glutaredoxin. The catalytic mechanism used by protein thiol-disulfide oxidoreductases for the formation or reduction of disulfides has been well characterized.[8] Briefly, the protein thiol-disulfide oxidoreductases catalyze disulfide exchange between the enzyme and the substrate via the formation of a mixed disulfide between the protein and the oxidoreductase (Figure 2). When an oxidoreductase catalyzes the oxidation of two substrate cysteinyl sulfhydryls, it becomes reduced. Likewise, when the oxidoreductase catalyzes the reduction of a disulfide bridge, it becomes oxidized. The redox state of the oxidoreductase can be regenerated by an enzyme, such as thioredoxin reductase or a small redox buffer, such as glutathione. The direction of the reaction catalyzed by the oxidoreductase is determined by its substrate and product concentrations, redox potential, and the redox conditions of the cellular milieu.

Members of the thioredoxin family of protein-thiol oxidoreductases have active sites with vicinal Cys residues in -Cys-Xaa-Xaa-Cys active site sequences (the thioredoxin active site sequence is -Cys-Gly-Pro-Cys).

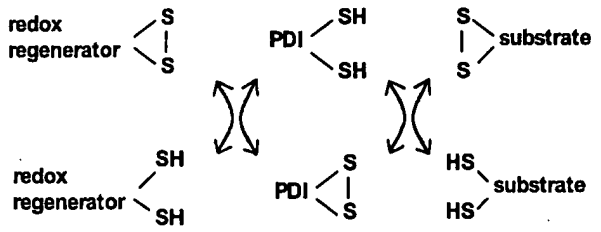

Figure 2. Enzymatic reaction catalyzed by protein-thiol oxidoreductases. The reaction catalyzed by protein-thiol oxidoreductases can be either a reduction of an existing disulfide, an oxidation of two thiols, or if more than one disulfide is present on the substrate protein, a disulfide isomerization. The regeneration of the oxidoreductase enzyme can be accomplished by a small thiol buffer, such as glutathione or another oxidoreductase.

Rat liver PDI has two active site Cys-Gly-His-Cys sequences at residues 35–38 and 379–382.[2] Each active site sequence contains two cysteines which can exist as reduced sulfhydryls, as a single oxidized disulfide, or as mixed disulfides with cysteinyl sulfhydryls of substrate polypeptide chains. Chemical modification of these cysteinyl sulfhydryls with iodoacetate leads to a complete loss of disulfide isomerase activity.[9] PDI is a unique member of protein thiol-disulfide oxidoreductases, capable of catalyzing both the oxidation and reduction of protein disulfides under physiologic conditions. The PDI active site redox couples are mildly oxidizing with a redox potential between −110 and −170 mV.[10] PDI in the lumen of the ER can act as both an oxidant and a disulfide isomerase. Conversely, when localized to the plasma membrane, cytosol, or endosomes, PDI acts as a reductant.

In the current model of PDI activity as a disulfide isomerase, the N-terminal Cys in the active site attacks a disulfide in the substrate forming a mixed disulfide with a substrate cysteinyl residue. The N-terminal Cys residue in each -Cys-Gly-His-Cys- PDI active site is a strong nucleophile when unprotonated under the physiologic conditions of the ER.[9] Once a mixed disulfide has formed between this reactive Cys residue and the substrate, the mixed disulfide is unstable due to the proximity of the C-terminal Cys of the PDI active site. Consequently, the vicinal Cys residues of the PDI active site reoxidize, forming a disulfide and releasing the displaced substrate with a reduced Cys free to reform another disulfide bridge, completing the isomerization reaction.

The structure–function relationships of the Cys-Gly-His-Cys active sites of PDI have been characterized by site-directed mutagenesis. Both Cys-Gly-His-Cys active sites (residues 35–38 and 379–382) are involved in PDI activity, as mutagenesis of either site causes a loss of 50% of the PDI activity and mutagenesis of both sites causes a complete loss of activity.[11] Mutagenesis of either the N-terminal or C-terminal Cys in each PDI active site causes a loss of PDI ability to achieve net oxidation or reduction of protein disulfides,[11,12] although only mutagenesis of the N-terminal Cys of each active site disrupts disulfide isomerase activity.[13] Despite the sequence and structural similarities between the a domains of PDI and thioredoxin, thioredoxin is 40-fold less active than PDI in assays of disulfide isomerase activity.[14] Furthermore, mutagenesis of the His residue within the PDI active site sequence (His-37) to Pro causes a significant loss in PDI activity.[15] This His37Pro muta-

tion generates PDI with an active site sequence identical to thioredoxin. Similarly, a Pro34His mutation of the active site of *E. coli* thioredoxin generates a mutant thioredoxin with redox characteristics more similar to PDI.[16] Therefore, it can be hypothesized that the active site Cys and His residues are directly involved in the PDI mechanism of action.

Small redox buffers can facilitate thiol disulfide exchange in the absence of enzymes, although the oxidoxreductases catalyze the oxidation or reduction of disulfides up to 10000 times faster.[17] Nevertheless, redox buffers may be important in regenerating the redox status of the oxidoreductase in order to maintain its catalytic activity. Some members of the protein-thiol oxidoreductase family, like glutaredoxin, are regenerated by a small thiol-disulfide buffer, such as glutathione, whereas others, like thioredoxin, are regenerated by another protein-thiol oxidoreductase. Thioredoxin reductase, the enzyme which regenerates thioredoxin redox status *in vivo*, can also regenerate the active site cysteines of PDI *in vitro* using $NADP^+$ and NADPH as the source of redox equivalents.[18] Although the system for maintaining the redox status of PDI has not been identified, PDI appears to prefer glutathione as a redox donor/buffer.[19] Glutathione (GSH) and its oxidized counterpart, GSSG, are the major electron donors and acceptors of the mammalian ER. The reducing environment of the ER is more oxidizing (GSH/GSSG between 2/1 and 3/1) compared to the reducing environment of the cytoplasm (GSH/GSSG between 30/1 and 100/1).[20] Recently, Ero1p has been identified as a candidate enzyme for the maintenance of GSH redox status in the ER.[21,22] Although Ero1p has not been identified as the direct mediator of the redox status of PDI, it does have an essential role in disulfide-mediated steps important to the kinetics of secretory protein folding.

Multi-domain structure is required for PDI activity

Despite the presence of oxidoreductase active sites in the a and a′ domains of PDI, the multi-domain structure of PDI is essential for its multiple redox activities. Early experiments demonstrating that mutation of either -Cys-Gly-His-Cys- causes a ~50% reduction in activity[11] led many to falsely conclude that the a and a′ domains were functionally equivalent. Although the sequence homology between the regions in the a and a′ domains surrounding the two active sites is remarkable, these redox sites are not functionally equivalent.[23] Mutagenesis of the C-terminal active site increases the K_m fourfold, while mutagenesis of the N-terminal active site has no effect on K_m. However, the N-terminal active site mutation decreases the k_{cat}, while the C-terminal active site mutation has no effect on k_{cat}. Consequently, at saturating concentrations of substrate, the C-terminal mutant retains it activity, while the N-terminal mutant has a significantly lower k_{cat}. The differences between the two sites may relate more to their proximity to the other domains of PDI rather than sequence differences within the domains themselves.

The functional interactions between the thioredoxin-like a and a′ domains and the other domains of PDI have only recently been investigated. The thioredoxin-like domains a and a′, can catalyze disulfide formation by themselves, but are unable to catalyze disulfide isomerization.[24] The acidic C-region of PDI is not required for either disulfide isomerase or chaperone activity, as truncation of the enzyme at residue 462 does not alter either activity.[25] A 21-kDa, C-terminal fragment of PDI (residues 308–491) comprising 1/3 of the b′-domain along with the a′ and c-domains displays the ability to oxidize and isomerize disulfide bonds on PDI substrates.[26] Because wild type PDI is a homodimer in the active state, it may be important that this 21-kDa fragment of PDI is a disulfide-linked dimer. The role of the non-thioredoxin-like domains in each of the PDI functions has also been carefully assessed using fragments of PDI generated by limited proteolysis and recombinant multi-domain constructs of PDI.[27] The b′ domain seems crucial for disulfide bond reduction as the a–b–b′ and b′–a′–c constructs were the smallest constructs capable of reducing BPTI. Similarly, b′–a′–c and a–b–b′ constructs were more effective in rearrangement of mixed disulfides between glutathione and BPTI. When assessing disulfide isomerase activity, b′–a′–c was the smallest PDI construct expressing isomerase activity. These results demonstrate the importance of the b′-domain in many of the redox activities of PDI, and coupled with the kinetic data, indicate that each domain contributes differently to the three types of protein-thiol oxidoreductase activity displayed by PDI.

PDI as a molecular chaperone

In addition to its activity as a protein-thiol oxidoreductase, PDI facilitates protein folding as a molecular

chaperone. At stoichiometric concentrations, PDI facilitates the refolding of denatured glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[28] and rhodanese.[29] Neither GAPDH nor rhodanese contains disulfide bridges, confirming the chaperone activity of PDI. The redox active sites of PDI are not essential to its chaperone activity, as alkylation of the active site Cys residues of PDI does not inhibit the refolding of denatured GAPDH.[30] Truncation of the C-terminal 51 amino acid residues of PDI causes a loss in chaperone activity, suggesting the peptide binding site located at the interface of the a′ and c domains (Figure 1) is essential to its chaperone activity.[31]

The evidence is quite convincing that PDI acts as both a protein-thiol oxidoreductase and a chaperone to facilitate protein folding *in vitro* and *in vivo*. In addition, when unfolded substrate is in vast excess over PDI concentrations *in vitro*, PDI facilitates the formation of large insoluble aggregates between itself and the unfolded polypeptides. This PDI function, termed anti-chaperone activity, has been demonstrated using both unfolded lysozyme[32] and unfolded alcohol dehydrogenase.[33] Anti-chaperone seems an appropriate term for this activity because the normal function of a chaperone is to limit aggregation of unfolded polypeptides. Anti-chaperone activity of PDI requires reducing conditions *in vitro*. When dithiothreitol is added to the medium in flasks of cultured cells, aggregates of chaperone-unfolded protein complexes are formed.[34] When the reducing agent is washed out of the cells, the unfolded polypeptides comprising these aggregates are salvaged and later appear as folded polypeptides secreted into the media.[35,36] Unfortunately, the role of PDI in the formation and salvage of these aggregates has not been elucidated. PDI does not display anti-chaperone activity with unfolded citrate synthase, indicating that like its disulfide isomerase and chaperone activities, there is some substrate specificity in PDI anti-chaperone activity.[33]

Multifunctionality

PDI is a subunit of P4H and MTP, two important enzymes in the post-translational modification and assembly of proteins. P4H catalyzes the co- and post-translational hydroxylation of nascent procollagen chains in the rough ER. All vertebrate P4Hs have been characterized as $\alpha_2\beta_2$ heterotetramers where the β-subunit is PDI. The β-subunit (PDI) continues to have oxidoreductase activity (including disulfide isomerase activity) as part of the P4H $\alpha_2\beta_2$ heterotetramer.[37] The P4H heterotetramer can self-assemble when the α- and β-subunits are co-expressed in insect cells[11] or in a cell-free system with dog pancreas microsomes.[38] Despite the presence of the substrate and co-substrate binding sites on the α-subunit of P4H,[39-41] the α-subunit is inactive and forms insoluble aggregates when it is expressed alone or dissociated from the β-subunit.[11,42] These results demonstrate that the β-subunit (PDI) functions as a chaperone, keeping the catalytic α-subunit in solution. However, the chaperone activity of the β-subunit is not its only function in P4H activity. Coexpression of the P4H catalytic α-subunit with BiP generates soluble α-subunit-BiP complexes with no P4H activity,[43] demonstrating that the β-subunit (PDI) has function in P4H activity other than its role as a chaperone.

Although the redox active sites of the PDI β-subunit are not directly involved in P4H function, the peptide binding site of PDI does bind P4H substrates, such as poly-proline and procollagen.[44] In fact, the peptide binding site of the β-subunit (PDI) is essential in the assembly of the P4H heterotetramer, as a deletion in that region (residues 452–454) totally abolishes P4H $\alpha_2\beta_2$ heterotetramer formation.[25] The affinity of P4H substrates for the peptide binding site is enhanced when PDI is a subunit of P4H, suggesting cooperativity of substrate binding between the α- and β-subunits of P4H.[44] Since the peptide binding site is required for PDI chaperone function[25,31] and the formation of P4H heterotetramer is enhanced by coexpression with procollagen,[45] these results may indicate that substrate binding by the β-subunit of P4H assists the formation of the heterotetramer. Procollagen binding may stabilize the subunits in an optimal conformation for $\alpha\beta$ subunit binding or orient them for enhanced subunit interaction. The PDI homodimer also participates in the folding and assembly of procollagen. Experiments utilizing semi-permeabilized cells suggest that P4H, HSP47, and PDI all participate (probably sequentially) in the folding and assembly of collagen (ref. 46 and Lamande and Bateman, this issue). Another function of the β-subunit (PDI) of the P4H complex is in subcellular localization of the P4H enzyme to the lumen of the rough ER. The presence of the carboxyl terminal -Lys-Asp-Glu-Leu (-KDEL) sequence on the β-subunit (PDI) localizes P4H to the ER.[47]

MTP facilitates the co-translational assembly of the

VLDL and chylomicron particles in the lumen of the ER of hepatocytes and intestinal cells by binding apoB co-translationally and allowing its lipidation. PDI forms a heterodimer with a 97-kDa protein to constitute the MTP complex.[48] A recently developed model of MTP structure predicts the N-terminal 300 residues of the large subunit of MTP form a β-barrel and the next 300 residues form a double-layered α-helical structure composed of 17 helices.[49] The surfaces of helices 15 and 17 of the C-terminal domain of the large MTP subunit form the interface with the PDI subunit of MTP.[49] The PDI subunit of the MTP heterodimer is essential to VLDL and chylomicron synthesis. A human subject with a genetic mutation causing the truncation of the C-terminal 30 amino acids of the 97-kDa α-subunit of MTP results in an inability to assemble the MTP heterodimer.[50] Intestinal biopsies from this patient demonstrate a complete absence of MTP heterodimer, resulting in an abetalipoproteinemia. MTP displays a high affinity interaction with apoB ($K_d = 10-30$ nM) that decreases with increasing lipidation of the apoB.[51] The affinity of MTP for apoB is much greater than homodimeric PDI, indicating binding cooperativity between the β-subunit (PDI) and the 97-kDa α-subunit of MTP. The N-terminal β-barrel domain of the large subunit of MTP (residues 22–303) binds the N-terminal region (residues 1–264) of apoB cotranslationally.[49] As the apoB polypeptide lengthens during translation, its affinity for the MTP complex decreases, with apoB18 having the highest affinity for MTP. ApoB also binds to helices 13–17 of the large subunit of MTP.[52,53] This apoB binding site overlaps with the large subunit-PDI binding site at helices 15 and 17, indicating simultaneous binding of apoB and PDI to the large subunit should be excluded. Bradbury *et al*[53] suggest that apoB displaces PDI from the MTP complex, lipid is transferred from MTP to apoB, then apoB dissociates from MTP to be replaced by PDI. MTP may function to retain unlipidated apoB in the ER or to maintain nascent apoB in a conformation that is able to be lipidated. However, inhibition of triglyceride synthesis in HepG2 cells decreases apoB-MTP interactions.[54] The PDI subunit of the MTP heterodimer functions as a chaperone rather than as an oxidoreductase, as mutagenesis of both redox active sites of the PDI subunit of MTP does not affect MTP activity *in vitro*[55] or secretion of apoB and triglyceride from cells expressing the mutant MTP.[56]

PDI also has the ability to covalently cross-link proteins through the formation of isopeptide bonds. This activity was identified during the cloning of transglutaminase from the filarial nematode *Dirofilaria immitis*. Transglutaminases (EC 2.3.2.13) catalyze the calcium-dependent covalent cross-linking of cellular proteins through the formation of ε-(γ-glutamyl)lysine isopeptide bonds. These covalent isopeptide bonds are important in the structural integrity of the sheath and cuticle of nematodes. Surprisingly, the isolated transglutaminase from *D. immitis* has 31–35% sequence homology with mammalian PDIs and PDI foldase activity.[57] Furthermore, bovine PDI demonstrates transglutaminase activity, suggesting that protein-thiol oxidoreductase and transglutaminase activity are present in many PDI enzymes. It is not known whether the relevant transglutaminase activity of this nematode occurs in the ER or at the surface of the parasite, although PDI was found in the excretory–secretory products of the nematode. In either location, PDI could play a crucial role in the production of cuticle proteins during the growth and maturation of this filarial parasite.

PDI is a major calcium binding protein of the ER, despite the absence of an EF hand calcium ion binding motif.[58] Many of the acidic residues in the PDI polypeptide (pI < 5.5) interact with calcium ion. The calcium binding affinity of PDI has been characterized as a weak interaction ($K_d = 2.8-5.2$ mM) with high capacity (up to 19 calcium ions bound per PDI).[59] Calcium binding by PDI results in conformational changes that are detectable spectroscopically.[59] It is not clear what role, if any, that calcium plays in PDI function. Initial reports indicated that unlike the ER chaperones calnexin and calreticulin, Ca^{2+} does not directly influence PDI-facilitated protein folding.[44,60,61] Millimolar concentrations of Ca^{2+} do not affect either the K_m or V_{max} of PDI-catalyzed folding of denatured ribonuclease A.[33] However, recent experiments suggest that Ca^{2+} induces conformational changes in sea urchin PDI (calcistorin) that affect substrate-specific PDI-facilitated folding.[62] Calcium may also regulate PDI activity by increasing its anti-chaperone activity. At low ratios of PDI to unfolded substrate, PDI displays anti-chaperone activity, causing the formation of large insoluble PDI-substrate aggregates. At higher ratios of PDI to unfolded substrate, PDI displays more chaperone activity and less anti-chaperone activity. Physiologic concentrations of Ca^{2+} present in the lumen of the ER favor PDI anti-chaperone activity.[33] Therefore, any Ca^{2+}-mediated modulation of PDI activity is probably through conformational changes which affect substrate interactions with the enzyme.

Substrate binding by PDI

PDI is a multifunctional enzyme with diverse activities including oxidation, reduction and isomerization of disulfides, chaperone and anti-chaperone activity, transglutaminase activity, and participation as a subunit of P4H and MTP complexes. The multifunctionality of PDI requires that the enzyme interacts with a broad spectrum of substrates undergoing processing in the ER. Additionally, PDI catalyzes redox reactions with proteins at the cell surface and in endosomes. PDI also interacts with cell surface integrins,[63] actin microfilaments,[64,65] and peptides transported into the ER by TAP for interaction with MHC I.[66,67] In all of these functions, a unifying characteristic of PDI appears to be its ability to interact with short stretches of polypeptide. The broad specificity of substrate interactions required for these activities might be explained by the presence of multiple substrate binding sites on PDI or by a single peptide binding site with little sequence specificity. Both hypotheses seem reasonable based on currently available data.

A PDI peptide binding site has been identified between residues 451–476 by affinity labeling with a hydrophobic tripeptide.[68] Subsequent experiments have further pinpointed the peptide binding site to a location between residues 452–461 in the a′-domain of PDI, partially comprising the last α-helix of that domain.[25] Saturation of the PDI peptide binding site with peptide affinity probe inhibits both disulfide isomerase and chaperone function.[69] However, truncation of the C-terminal 51 residues of the PDI molecule (including the proposed peptide binding site) inhibits peptide binding and chaperone activity, but does not eliminate disulfide isomerase function.[31] Although these results indicate that the peptide binding site is not essential to disulfide isomerase activity, a complete kinetic analysis of the truncated PDI has not been performed. Recent experiments assessing the peptide binding activity of recombinant constructs of PDI have demonstrated the importance of the b′ domain of PDI in small peptide binding.[70] In fact, the b′ domain is sufficient for binding small peptides, such as somatostatin and mastoparan, although binding larger polypeptides, such as the PDI substrates BPTI and ribonuclease A require the b′, a′, and c domains.

Recent evidence indicates that PDI is the primary acceptor of peptides translocated into the ER. The MHC class I-mediated immune response requires the transport of cytosolic peptides into the ER through the ER transmembrane transporter TAP. Once in the lumen of the ER, peptides bind to PDI, gp96, calreticulin, gp120, gp170, or the MHC class I molecules, with PDI as the dominant acceptor.[66,67] Unlike other ER chaperones, PDI displayed no identifiable sequence specificity for peptide binding.[67] It is not clear whether peptides bound by PDI are transferred to the class I MHC, although it has been suggested that another ER chaperone, gp96, has a direct role in peptide loading of MHC class I.[71]

Subcellular localization of PDI

The site of translation, folding, and the earliest steps in post-translational modification and subunit assembly occur in the lumen of the rough ER. The soluble ER chaperones and foldases, including PDI, are retained in the ER lumen by a -KDEL (-HDEL in yeast) sequence at their carboxyl terminus. KDEL proteins like PDI are recycled from the cis Golgi network by binding to a -KDEL receptor and returning to the ER from the Golgi via a retrograde pathway (reviewed in ref. 72). Ca^{2+} binding by PDI may also contribute to the retention of PDI in the ER through an unidentified mechanism. Addition of the calcium ionophore A23187 or the calcium pump inhibitor thapsigargin causes the release of PDI from the ER.[73] Experiments in our laboratory with liposomes generated from ER lipids indicate that Ca^{2+} -mediated ER retention of PDI does not require any ER membrane protein.[44]

PDI is also found in the rest of the endomembrane system including the Golgi, secretory vesicles, and ultimately, the plasma membrane.[74] PDI is diffusely localized in the cytosol of some cells not containing identifiable ER, such as leukocytes and megakaryocytes, although the concentrations of cytosolic PDI in these cells is significantly lower than levels of ER-localized PDI found in other cells.[75] PDI has also been found localized to the cytosol of some cell types containing ER. A significant amount (25%) of the total cellular PDI of glial astrocytes is present in the cytosol.[76] Upon exposure of glial cells to thyroxine, the cytosolic PDI redistributes almost completely to become actin-associated, suggesting PDI acts as a mediator of thyroxine-induced actin nucleation.[64,65] Human polymorphonuclear neutrophils (PMNs), the major cellular component of the acute inflammatory response, contain PDI despite having little or no ribosomes or ER.[77] The PDI in PMNs is associated with storage granules, suggesting that PDI may function in the activation of latent proteinases.

PDI localizes to the plasma membranes of hepato-

cytes and pancreatic cells despite possessing an intact -KDEL ER retention signal.[74,78-80] Secretory cells easily saturate the -KDEL retrieval mechanism, resulting in the non-covalent binding of secreted PDI to the plasma membrane. The appearance of the secreted PDI at the cell surface may have significant physiological effects. First, localization of PDI to the plasma membrane may elicit an autoimmune response. The Long Evans Cinnamon rat displays a spontaneous hereditary hepatitis and hepatic carcinoma. The autoimmune antibodies generated in the progression of this hereditary hepatitis bind PDI and calreticulin.[81] Anti-PDI antibodies are also found in humans with liver disease and chronic alcoholism.[82] Second, membrane-associated PDI may participate in the reduction of membrane receptors and/or ligands. PDI is a functional protein-thiol oxidoreductase on the surface of B-lymphocytes.[83] In fact, cell surface levels of PDI correlate with the expression of free thiols on surface proteins of the B-lymphocytes and B cell chronic lymphocytic leukemia cells.[84] Inhibition of surface PDI activity with bacitracin or anti-PDI antibodies results in increased levels of surface free thiols. Lymphocytes treated with bacitracin are less sensitive to cytostatic drugs, such as chlorambucil, vinblastine, and cisplatin, suggesting surface PDI activity increases susceptibility to some cytostatic drugs. These observations indicate a physiologic role for surface PDI that may be useful in the treatment of B-cell leukemias.

The protein-thiol oxidoreductase activity of membrane associated PDI is required for the activation of diphtheria toxin. Diphtheria toxin is a heterodimer composed of a catalytic subunit and a receptor binding subunit linked by disulfide bridges. The toxin heterodimer must be cleaved after binding to the plasma membrane in order to be activated. This reductive cleavage of the toxin into two polypeptides is mediated by cell surface PDI.[85] After reductive cleavage, the catalytic subunit is translocated across endosomal membranes and the membrane binding subunit incorporates into the membrane. Similarly, membrane PDI inactivates insulin internalized during the endocytic process by reduction of the intermolecular disulfides between the A and B chains. Unlike diphtheria toxin, cholera toxin is activated by PDI in the ER.[86,87] Cholera toxin is activated by the reduction of its A subunit to the A_1 peptide. The A_1 peptide is an ADP-ribosyltransferase that activates the α-subunit of the stimulatory G-protein of adenyl cyclase.

PDI may also be active as a soluble protein-thiol oxidoreductase outside of the cell. Platelets secrete PDI, some of which localizes to the plasma membrane and remains active.[75] Although platelets have no identifiable ER, internal PDI is diffusely localized in the cytosol and at the cell surface. Platelets activated by thrombin or the calcium ionophore A23187 secrete PDI into the surrounding media.[88,89] The PDI secreted by the activated platelets is apparently released from its internal cytosolic localization by vesiculation. PDI secreted by platelets is active in *in vitro* assays, suggesting that it may have a significant physiological role in protein-thiol mediated events in the region of the wound. PDI catalyzes a disulfide isomerization reaction in thrombospondin 1 and its 120-kDa fragment in the calcium binding region of the molecule, which may affect their interaction with thrombin.[90,91]

Cell surface PDI may participate in cell signalling through interactions with membrane receptors. The shedding of extracellular domains of membrane receptors is a mechanism of regulating the activity of many types of receptors, including receptors for interleukins, transferrin, insulin, tumor necrosis factor, thyroid stimulating hormone (TSH), insulin growth factor, and colony stimulating factor I (reviewed in ref. 92). Membrane PDI catalyzes the shedding of the extracellular domain of the TSH receptor in the thyroid gland.[93] Reagents which inhibit PDI activity, such as antibodies against PDI, the cell impermeant sulfhydryl reagent DTNB, and bacitracin, increase TSH receptor levels on the plasma membrane by decreasing receptor shedding. These results indicate that the thyroid gland may regulate receptor activity by controlling levels of membrane associated PDI. However, the extent to which surface localization of PDI regulates other types of receptors is unclear at this time.

Plasma membrane PDI may also regulate cellular adhesion. Integrins expressed at the surface of leukocytes are required for extravasation, migration, and adherence to extracellular matrix. The ability of leukocytes to adhere may be a result of activation of latent surface integrins rather than increases in integrin expression. Recent evidence indicates that PDI is required for the activation of β_1 and β_2 integrins on activated lymphocytes.[63] Plasma membrane PDI may also be involved in the process of contact inhibition of fibroblasts. Intracellular PDI levels increase in normal fibroblasts as they approach confluency. Addition of interferon-β to HT1080 fibrosarcoma cells induces PDI gene transcription and restores contact inhibition.[94] Furthermore, bacitracin inhibits the

ability of interferon-β treated cells to regain contact inhibition. These results demonstrate the importance of cell surface PDI to the regulation of cell–cell interactions.

Regulation of PDI gene expression

The cDNA sequences encoding PDI have been cloned in a number of organisms and demonstrate a high level of sequence similarity. The human PDI gene (GenBank #J04049 and J04050) is 16.5 kb divided into 11 exons. The 5′ flanking region of the human PDI gene contains a TATA box, six CCAAT boxes between −108 and −378, two GC-rich regions, and four variations of the decanucleotide consensus sequence for binding Sp1.[95] This 5′ flanking region shares extensive homology with the ER chaperones calreticulin, BiP, and GRP94, indicating similar transcriptional regulation of these four genes.[96] PDI is found abundantly in almost all tissues, with PDI mRNA levels correlating with tissue expression of the enzyme.[97] Heat shock, A23187, tunicamycin, cycloheximide, dithiothreitol, and Δ^{12}-prostaglandin J_2 induce PDI transcription, although PDI levels seldom increase greater than three- to fourfold.[98] PDI is transcriptionally upregulated in the brain and liver of the developing rat late in fetal development.[99] However, PDI mRNA levels are not significantly altered between 10- and 20-day old, and adult mice in any tissue investigated except for the pituitary.[97] These results indicate that PDI is induced in early development during times when large amounts of protein are being synthesized, but PDI transcription levels are relatively stable after birth. Regulation of PDI expression may also control PDI function by altering the subcellular localization of the PDI enzyme. The -KDEL retrieval mechanism is easily saturated, resulting in significant redistribution of PDI from the ER to the cell surface during stress events. Because the amount of PDI which escapes the ER retrieval system is small in the uninduced state, the differences in PDI localized to the plasma membrane are altered significantly upon transcriptional activation of the PDI gene. A three- to fourfold transcriptional activation of PDI might therefore result in a several fold induction of membrane PDI expression.

PDI expression is regulated transcriptionally by several hormones, including insulin. Levels of both PDI mRNA and PDI are increased threefold in the livers of diabetic rats.[100] Moreover, insulin treatment of diabetic rats reverses this effect, with PDI mRNA transcription decreasing within 30 min. Stimulation of the pancreas *in vivo* with cholecystokinin or the secretogogue cerulein leads to 2.5- to fourfold increases in PDI, BiP, calreticulin, and hsc mRNA, indicating coordinated regulation of these chaperones.[101] Although the expression of ER chaperones is often coordinately regulated, long-term dietary changes do not affect expression of all ER chaperones equally. Decreasing caloric content of the diet by 50% decreases ERp57, ERp72, BiP, GRP94, GRP170, calnexin, and calreticulin expression in liver, but has no effect on PDI, HSC70, and GRP75 levels.[102] This dietary effect on the expression of selected chaperones is found only in liver, indicating the mechanism involves liver-specific factors or relates to the liver's unique role in serum glucose regulation.

Retinoic acid-induced differentiation of the SH57 neuroblastoma[103] and F9 teratocarcinoma cell line[104] induces transcription of PDI mRNA. Furthermore, bacitracin, an inhibitor of PDI activity, abrogates the retinoic acid-induced differentiation of the SH57 cell line.[103] These results indicate that the expression of PDI during retinoic acid-induced cellular differentiation is a common, controlled, and likely essential event. Similarly, induction of cultured mouse spleen lymphocytes and BCL_1 cells with various mitogenic agents, such as lipopolysaccharide, causes a significant increase in cellular PDI levels.[105] Other factors which induce lymphocyte excitation or mitogenesis without differentiation into Ig-secreting lymphocytes have no significant effect on PDI levels. Therefore, induction of PDI expression may correlate more with differentiation of lymphocytes than activation.

Reports have also demonstrated the possible use of PDI for clinical diagnostics. PDI is one of the 32 proteins induced (fourfold) in human breast ductal carcinoma.[106] PDI may also be elevated in tissues of women at risk for preterm deliveries. Quantitation of PDI mRNA levels in amnion, chorion, and placenta indicates PDI levels are higher in women who underwent pre-term labor (< 30 weeks).[107] Although it is not understood why PDI levels are elevated in the placentas of women who underwent pre-term labor, the authors hypothesize that oxidative stress may induce PDI expression and contribute to the pathophysiology of the pre-term labor. However, oxidative stress and Ca^{2+} agonists do not increase PDI expression in cultured umbilical vein endothelial cells.[108]

Recent evidence has suggested genes can be regulated by protein-thiol oxidoreductase control of gene expression. The redox status of thiols on transcriptional factors appears to be a target for a regulatory

mechanism for transcriptional activation similar to regulation by phosphorylation/dephosphorylation. Several mammalian transcription factors, such as NF-κB and AP-1 appear to be controlled by oxidation and reduction of key disulfides (reviewed in ref. 109). In fact, simple changes in the ratio of reduced to oxidized glutathione significantly affect NF-κB and AP-1 binding to DNA as assessed by electromobility shift assays.[110] Furthermore, transcription factor binding is also controlled by PDI *in vitro*. PDI shifts the binding ability of NF-κB and AP-1 in opposite directions and has no effect on the DNA binding of Oct1, SP1, and TFIID. Although it is not clear how (or where) PDI would regulate nuclear transcriptional factors *in vivo*, these results indicate PDI-facilitated redox control of transcription is a plausible modulator of gene expression. PDI also regulates gene expression through the redox control of translation. Chloroplast PDI regulates the expression of the *psbA* gene in *Chlamydomonas reinhardtii* by redox control of the RB47 mRNA binding protein.[111] In this mechanism of PDI-controlled translational activation, light turns on the RB47 mRNA binding protein by activating chloroplast PDI. Light activates chloroplast PDI through an electron transfer relay system involving ferredoxin and ferredoxin-thioredoxin reductase. PDI activates RB47 through the reduction of an RB47 disulfide, resulting in the activated RB47 binding to the 5′-untranslated region of the *psbA* mRNA. Redox-based regulation of gene expression is an interesting mechanism of gene control at either the transcriptional or translational level. However, further investigation will be required to demonstrate that PDI is the physiologically relevant oxidoreductase controlling gene expression in other systems.

Acknowledgements

The author thanks Michael Swartz for critical review of this manuscript. RN is supported by a CAREER grant MCB9631485 from the National Science Foundation.

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